채서현 석사 학위논문

Comparative analysis of plasma and salivary cortisol levels in canine adrenal insufficiency models

개의 부신 기능 부전 모델에서 혈장 및 타액 내 코티솔 수치의 비교 분석

2020 년 8 월

서울대학교 대학원

수의학과 임상수의학 전공

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이 논문을 채서현 석사 학위논문으로 제출함

2020 년 5 월

서울대학교 대학원

수의학과 임상수의학 전공

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채서현의 석사 학위논문을 인준함

2020 년 7 월

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Comparative analysis of plasma and salivary cortisol levels in canine adrenal insufficiency models

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ABSTRACT

Objectives: This study aimed to measure both plasma and salivary cortisol levels using a commercial solid-phase, competitive chemiluminescence enzyme immunoassay system and to investigate the correlation between plasma and salivary cortisol levels in dogs with low plasma cortisol levels (hypoadrenocorticism).

Materials and Methods: Using a commercial chemiluminescence immunoassay (Immulite 1000), cortisol levels in plasma and saliva of healthy Beagle dogs (n = 10) were measured serially, following a low-dose dexamethasone suppression test. Plasma and salivary cortisol concentrations were then analysed for the presence of a direct correlation under low endogenous cortisol levels.

Results: A significant linear correlation was observed between the plasma and salivary cortisol concentrations measured in the dexamethasone suppression model.

Clinical significance: Cortisol is a stress-reflecting hormone. Therefore, the cortisol assay is influenced by how stressful the method of sample collection is, i.e. whether it is invasive (venepuncture) or non-invasive (saliva collection). The current study demonstrated that the assessment of salivary cortisol can be considered to obtain physiologically relevant information of plasma cortisol in patients with hypoadrenocorticism, using a convenient commercial immunoassay.

Keywords: chemiluminescence immunoassay, canine hypoadrenocorticism, non-invasive assessment, stress hormone.

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INTRODUCTION

Cortisol is a steroid hormone secreted primarily from the adrenal cortex in response to stress. This glucocorticoid is part of an intricate neuroendocrine feedback mechanism within the hypothalamic-pituitary-adrenal (HPA) axis. Cortisol is released peripherally into all bodily fluids including the blood, spinal fluid, semen, urine, and saliva. It is involved in a myriad of physiological processes such as immunosuppression, blood pressure maintenance, and the regulation of glucose storage and expenditure. Disturbance in cortisol homeostasis causes an assembly of clinical symptoms, depending on the degree of change in cortisol concentration (Besser *et al.* 1976). As an important indicator of adrenocortical function, anomalous increase or decrease in cortisol levels indicates the presence of Cushing's syndrome or Addison's disease, respectively (Besser *et al.* 1976). Differential diagnoses of these endocrine disorders have traditionally been performed by serial measurements of blood (serum or plasma) cortisol levels following adrenocorticotropic hormone (ACTH) stimulation, or an exogenous glucocorticoid suppression test (Thompson *et al*., 2007; Vincent & Michell 1992). However, it has been debated as to whether invasive sampling via venepuncture causes iatrogenic stress, subsequently raising cortisol levels.

In human medicine, salivary cortisol measurement has become a major replacement to blood cortisol measurement, owing to the non-invasive nature of sample collection (Beerda *et al.* 1996). Although much still remains unclear regarding the use of salivary cortisol in animals, the increase in the incidence of companion animal metabolic disorders has led researchers to focus on non-invasive and non-iatrogenic collection of salivary cortisol from animals. Measuring salivary

cortisol has benefits such as sampling in a stress-reducing environment (e.g. self-sampling in humans or sampling by the dog's owner), an accurate representation of the bioactive fraction unbound to cortisol-binding globulin (CBG) (Coolens *et al.* 1987), a reliable depiction of diurnal fluctuation in cortisol secretion, and a significantly shorter sample collection time in comparison to a 24-h urinary cortisol collection (Koyama *et al.* 2003).

Although previous studies in humans analysed data using both manual and fully automated immunoassays, results from canine studies are limited to manual assays including enzyme immunoassay (EIA), radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA). Automated assays reduce intrinsic errors inevitable in manual assays and improve the precision of the overall system (Allinson, 2011). Results from human studies demonstrated a significant association between plasma and salivary cortisol levels under normal, hyperadrenocortical, and hypoadrenocortical conditions (Shimada *et al.* 1995; Jung *et al.* 2014). Studies in different animal species, including domestic dogs, have demonstrated a similar correlation between cortisol in plasma and saliva as in humans, but results were limited to normal or hyperadrenocortical patients (Wenger-Riggenbach *et al.* 2010; Escribano *et al.* 2012; Menargues *et al.* 2012; Cobb *et al.* 2016).

To the authors' knowledge, the present study is the first to measure both plasma and salivary cortisol levels using an automated commercial solid-phase, competitive chemiluminescence enzyme immunoassay system and aim to elucidate the correlation between plasma and salivary cortisol levels in dogs with low plasma cortisol levels (i.e. hypoadrenocorticism). To achieve this, healthy dogs underwent a low-dose dexamethasone suppression test (LDDST) to temporarily suppress endogenous cortisol secretion (Tiller *et al.* 1988). It was hypothesised that a linear

correlation would exist between plasma and salivary cortisol in dogs whose cortisol secretion is suppressed following LDDST.

MATERIALS AND METHODS

Ethical approval

The study protocol was approved by Seoul National University Institutional Animal Care and Use Committee (SNU-191017-5).

Sample collection

Ten purpose-bred, Beagle dogs (9 males, 1 female), housed at the laboratory animal facility at the College of Veterinary Medicine, Seoul National University, were used. The dogs underwent complete physical and hemodynamic examinations to ensure that they were clinically and hemodynamically stable. The dogs were aged between 1 to 5 years, weighed between 8 and 12 kg, and had a normal body condition score (BCS) of between 4 and 5, out of 9, based on the study by German *et al.* (2006).

On day 1 (endogenous control day), baseline plasma and salivary cortisol samples were collected from all 10 dogs at one-hour intervals (T0, T1, T2, T3, T4, and T5), starting at 8:30 a.m. (T0) until 1:30 p.m. (T5). On the second sample collection day, (LDDST day), two weeks after the endogenous control day, when the dogs were assumed to be free from any stress associated with the previous sample collection, the same 10 dogs were injected with 0.01 mg/kg intravenous dexamethasone (Hanall, Seoul, South Korea) to induce suppression of cortisol release (i.e. LDDST), for at least 4 h. Sampling times were identical to those of the endogenous control day, except that the first sampling was performed immediately after the dexamethasone injection (Harris *et al.* 1990).

Dogs were minimally restrained during sampling. Blood was collected in a heparin tube via venepuncture of the jugular vein and was centrifuged at 4,500 × *g*

for 2 min to separate plasma. Saliva was collected within 2 min of venepuncture using sterile cotton balls placed between the upper and lower premolars for 2 min, held with a pair of clamps. Salivation was stimulated by placing an open can of dog food within sight of the dog (Phillips *et al.* 1983). Saliva-immersed cotton balls were placed in a plain tube and were centrifuged at 4,500 × *g* for 25 min. Before each saliva collection, the mouth was rinsed with water injected by a sterile plastic syringe.

A minimum of 300 μl of plasma and saliva was collected during each session. Eleven samples that did not reach the minimum required volume for the assay were excluded. Samples were stored at −80 ºC until the assay was done (Gozansky *et al.* 2005).

Assay

Total plasma cortisol was measured using a commercial chemiluminescence immunoassay system (Immulite 1000 Siemens Healthcare Diagnostics, Deerfield, IL, USA). Salivary cortisol was extracted with ethyl acetate and measured using an identical assay.

Statistical analysis

Statistical analyses were performed using a commercial statistic software (SPSS version 25.0, IBM, Chicago, IL, USA), and the product-moment correlation coefficient (Pearson's correlation coefficient) was used to establish the correlation between the cortisol concentrations in the plasma and saliva. A P value of less than 0.05 was considered to represent statistical significance.

RESULTS

Cortisol in all samples was measured using a commercial chemiluminescence immunoassay system (Immulite 1000). The analytical sensitivity was 0.108 $\mu g/dl$ (3 nmol/l). The inter-assay coefficients of variation (CV) at each cortisol concentration are listed in Tables 1 and 2.

Baseline plasma cortisol values ranged from 1.84 to 9.64 $\mu g/dl$ (50.77–265.97 nmol/l), and saliva cortisol varied from 1.0 to 1.59 $\mu g/dl$ (27.59–43.87 nmol/l) (Table 1).

The concentrations of plasma and salivary cortisol after the administration of low-dose dexamethasone are summarised in Table 2 and Fig. 1. Minimum values after dexamethasone administration in plasma and saliva cortisol were observed at measuring points T2 and T3 in all dogs (Fig. 1).

Baseline salivary cortisol concentrations were on average 13.91% of the baseline plasma cortisol concentrations. Following the LDDST, salivary cortisol concentrations were on average 20.65% of the plasma cortisol levels.

After the LDDST, cortisol secretion (in plasma and saliva) was suppressed to 13.90% and 18.65%, respectively, compared to the basal levels.

Saliva concentrations were on average 17.89% of plasma concentrations of cortisol in the dexamethasone-suppressed group. The correlation between cortisol in the saliva and plasma after LDDST was significant at $R_2 = 0.920$ ($P < 0.01$, Fig. 2).

Table 1. Mean changes in cortisol concentration (µg/dl) at each time point on endogenous control day.

		T0	T1	T2	T3	T4	T5
Plasma	Mean	4.802	5.813	5.96	5.92	5.517	5.418
	±SE	0.537	0.511	0.518	0.418	0.669	0.483
	CV (%)	33.5	25.1	24.9	21.2	36.5	26.7
Saliva	Mean	1.28	1.35	1.348	1.345	1.285	1.259
	±SE	0.054	0.053	0.067	0.056	0.065	0.055
	CV (%)	12.6	11.9	14.8	12.5	15.2	13.1

SE, standard error; **CV**, coefficient variant

Table 2. Mean changes in cortisol concentration (µg/dl) following injection of dexamethasone at each time point.

		T0	T1	T2	T3	T4	T5
Plasma	Mean	0.814	0.799	0.788	0.779	0.83	0.844
	±SE	0.019	0.013	0.009	0.02	0.019	0.017
	CV (%)	0.071	0.05	0.034	0.076	0.079	0.066
Saliva	Mean	0.198	0.161	0.15	0.134	0.182	0.226
	±SE	0.021	0.015	0.02	0.018	0.015	0.02
	CV (%)	0.308	0.25	0.302	0.303	0.202	0.317

SE, standard error; **CV**, coefficient variant

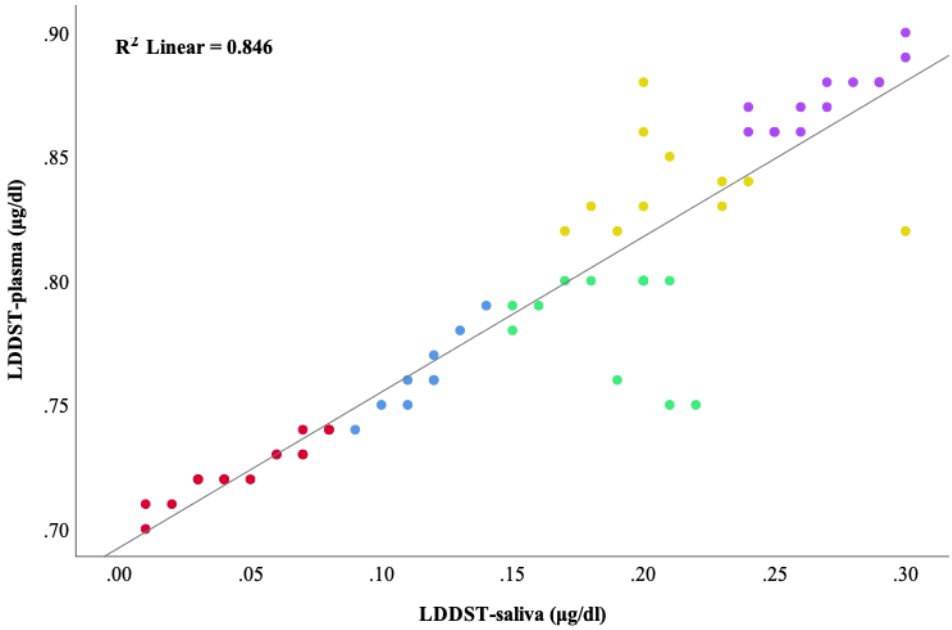

Fig. 1.

Fig. 1. Total plasma and salivary cortisol levels following low-dose dexamethasone suppression test (LDDST) (µg/dl)

Legend
Purple dots: sampled at T1
Red dots: sampled at T2
Blue dots: sampled at T3
Green dots: sampled at T4
Yellow dots: sampled at T5

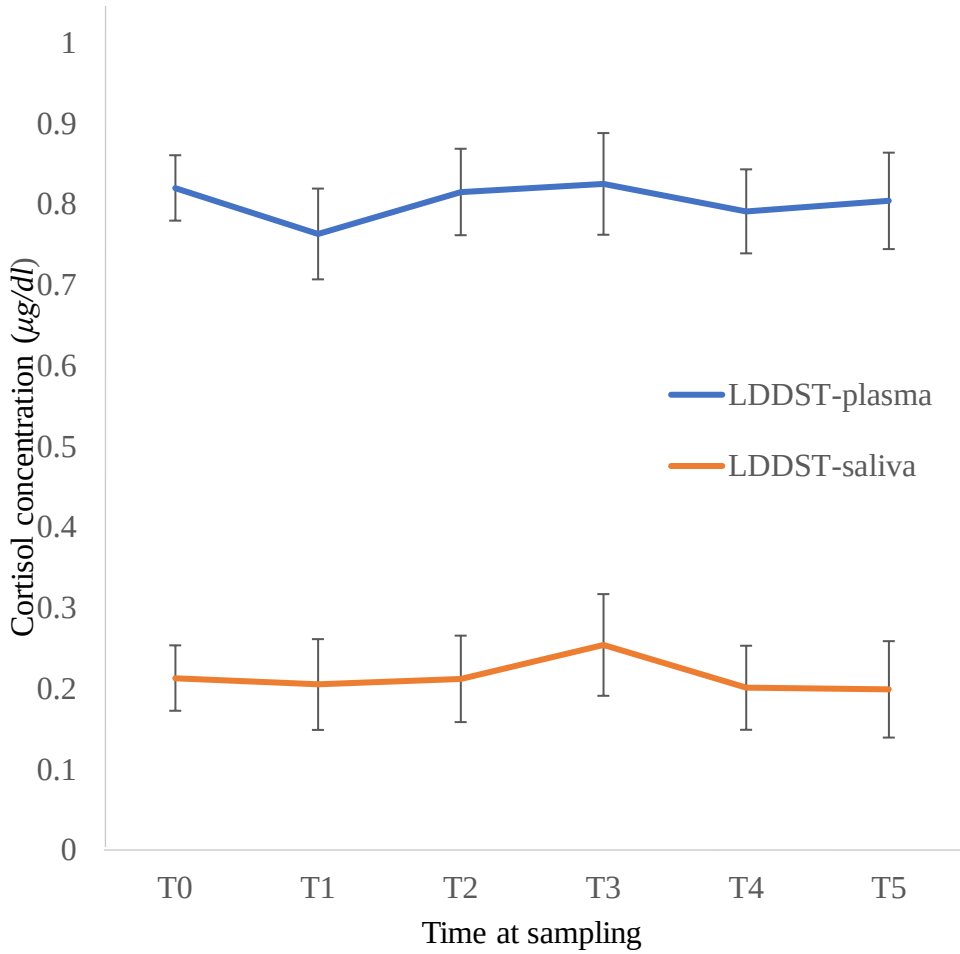

Fig. 2. Changes in mean plasma and salivary cortisol (μg/dl) measured at 1 h intervals following low-dose dexamethasone suppression test (LDDST).

DISCUSSION

The aim of this study was to measure plasma and salivary cortisol following exogenous glucocorticoid suppression of endogenous cortisol secretion using a commercial chemiluminescence immunoassay system, and to analyse correlations between cortisol in the two media during a hypoadrenocortical condition. Previous canine studies on the association of cortisol in different body fluids were limited to patients with normal or elevated cortisol concentrations (El-Farhan *et al.* 2017, Tvarijonaviciute *et al.,* 2018). Additionally, these studies were all performed using manual assays, such as EIA, RIA, or ELISA (Cobb *et al.* 2016). Therefore, to our knowledge, this study is the first to provide a direct comparison of plasma cortisol with simultaneously obtained salivary cortisol samples, under cortisol suppression treatment, using a commercial chemiluminescence immunoassay system (Immulite 1000).

In terms of the methodology adopted in the present study, Immulite 1000 is a preferred assay in human research for evaluating salivary cortisol, in comparison to other manual assays. However, its availability has been limited to overseas clinical pathology laboratories (e.g. IDEXX) or university hospitals in the veterinary field. In the present study, this system was readily accessible for immediate analysis and was selected for its equivalent accuracy (analytical sensitivity and specificity) and improved measurement convenience compared to other manual assays (Allinson, 2011). Data obtained from the current study also support the reliability of the present methodology because baseline cortisol concentrations (1.0 to 1.59 $\mu g/dl$) were within the range of previously published data (0 to 33.79 $\mu g/dl$) obtained using EIA, RIA, and ELISA, assembled in the meta-analysis of Cobb *et al.* (2016). Our results suggest

that a strong, linear correlation (R_2 = 0.920, P < 0.01) exists between plasma and salivary cortisol over periods of suppressed cortisol secretion.

The authors adopted and modified the study design from human models for glucocorticoid replacement therapy in adrenal insufficiency (Howlett 1997; Jung *et al.* 2014; Wong *et al.* 2004). LDDST was successfully used in the creation of a canine hypoadrenocortical model as both plasma and salivary cortisol concentrations remained below 1.0 $\mu g/dl$ throughout the experiment. This protocol was also adopted in an attempt to establish the subjects' endogenous basal cortisol concentrations as a control prior to treatment with LDDST by using the same minimised inter-individual variances. The magnitude of change in plasma cortisol (13.90% of baseline value), and the magnitude of the relative change in salivary cortisol (18.65% of baseline value) did not show a significant difference after LDDST (Gozansky *et al.* 2005). This degree of change agreed with a previous human study on the comparison of serum and salivary cortisol using an EIA (Raff *et al.* 2003). In the study by Gozansky *et al*. (2005), salivary cortisol concentrations increased at a higher rate compared to plasma cortisol following vigorous exercise and oral oestrogen administration, while no difference was demonstrated between saliva and plasma cortisol following dexamethasone suppression. This suggests that the dynamic changes in the free fraction of cortisol within saliva are better observed during increased cortisol secretion when the response to HPA stimuli exceed the binding capacity of CBG (Brien 1981; Scott *et al.* 1990). Additionally, the time course of the response was similar in both media, reaching minimum levels 2 and 3 h following LDDST, suggesting that salivary cortisol also changed in accordance with the diurnal rhythm of the HPA axis, concurrent with circadian changes in plasma cortisol (Koyama *et al.* 2003; Newell-Price *et al.* 2008).

A meta-analysis by Cobb *et al.* (2016) on canine salivary cortisol corroborated a plethora of factors which influence the measurement of cortisol in saliva, including endogenous features (sex and neuter status, age, and living conditions) and methodological factors (testing environment, owner presence during testing, collection media, and use of salivary stimulant). However, in the present study, a causal inference was not made between plasma and salivary cortisol concentrations using a regression analysis, as cortisol was collected and analysed under identical influences (i.e. covariates) for both media, in an attempt to minimise the influence from external factors other than the treatment (LDDST).

Measurement of salivary cortisol has several advantages over plasma cortisol measurement (Paraa *et al.* 2005). The stress of venepuncture can be avoided, especially in aggressive/sensitive and debilitated patients with difficult access to veins, and samples can be collected in a more familiar environment (e.g. at-home sampling by the owner) (Kahn *et al.* 1988; Vining *et al.* 1983). Additionally, only the bioactive fraction of the hormone was measured. Furthermore, with the methodology used to assess plasma and salivary cortisol in the present study, the sample did not require incubation, and the results were available within an hour of sample collection (Lennon *et al.,* 2007; Meeran *et al.* 1993). Moreover, because cortisol remains moderately stable within plasma or saliva samples for months (Chen *et al.* 1992; Damian *et al.* 2018), referring to a clinical pathology laboratory with an identical assay machine would be a reasonable option. In terms of the stress-reducing nature of the salivary sample, the authors used minimal restraint and rejected the idea of intramuscular sedatives used by Phillips *et al.* (1983). Sufficient sample was obtained in the current study using cotton balls and spontaneous salivation after visual stimulation with dog food in most cases.

There are a few limitations to this study. Firstly, this was an exploratory/discovery study to investigate salivary cortisol concentrations under suppressed cortisol secretion conditions. Therefore, further research is necessary to reach a consensus on the reference range for salivary cortisol concentrations among various assay methodologies. Secondly, our results should be considered preliminary because the models of hypoadrenocorticism used in this study do not represent all of the diagnostic requirements of canine Addison's disease, due to the absence of clinical signs, and electrolyte derangements. In future studies, salivary cortisol assessments should be considered in actual Addison's patients that have been diagnosed with other modalities, including medical history, clinical signs, physical examination, ACTH stimulation test, electrolyte panels, and abdominal ultrasonography to provide more clinically relevant data.

In summary, we have demonstrated the non-invasive assessment of salivary cortisol with a simple, commercially available chemiluminescence immunoassay system, and the salivary cortisol levels were found to have a direct relationship with total plasma cortisol in dogs with hypoadrenocorticism. This finding has important clinical implications in the diagnosis of canine hypoadrenocorticism. The assessment of salivary cortisol should be considered for patients suspected of having hypoadrenocorticism to obtain a more physiologically relevant and accurate diagnosis.

Conflict of interest

No conflicts of interest have been declared.

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국 문 초 록

개의 부신 기능 부전 모델에서 혈장 및 타액 내 코티솔 수치 측정

코티솔은 스트레스를 반영하는 호르몬이다. 따라서 코티솔 분석은 시료 채취 방법에서의 스트레스 정도, 즉 침습성 (채혈) 인지 비침습성 (타액 채취) 인지에 영향을 받는다.

본 연구는 상용 고체상 경쟁화학발광효소면역측정법 (solid-phase, competitive chemiluminescent enzyme immunoassay)을 사용하여 혈장 및 타액의코티솔 농도를 측정하고, 혈장 코티솔 농도가 낮은 개에서 혈장과 타액 코티솔 농도 사이의 상관 관계를 조사하는 것을 목표로 했다.

상용 화학발광면역측정법을 사용하여, 저용량의 덱사메타손 억제 시험을 진행한 건강한 비글개 (n = 10)의 혈장 및 타액에서의 코티솔 농도를 연속적으로 측정하였으며, 혈장 및 타액 코티솔 농도 사이에 유의한 선형 상관이 관찰되었다.

본 연구에서 부신 호르몬 분비가 억제된 개의 타액과 혈장 코티솔 수치는 강한 상관 관계를 보였다. 따라서, 정확한 정보를 얻기 위해서는 의인성 스트레스의 영향을 감소시킬 수 있는 타액 코티솔의 평가가 혈장 코티솔보다 선호될 수 있을 것으로 고려된다.

주요어 : 부신기능부전, 타액 코티솔, 화학발광면역분석
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